Author's response to reviews

Title: Influence of type 2 diabetes on local production of inflammatory molecules in adults with and without chronic periodontitis: a cross-sectional study

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Author's response to reviews: see over
Dear Prof. Deschner,

On behalf of the authors, I would like to thank you and the reviewers for the thoughtful comments, and for the time and expertise that have been invested in reviewing our manuscript “Influence of type 2 diabetes on local production of inflammatory molecules in adults with and without chronic periodontitis: a cross-sectional study”, (MS: 1403847891166203). The comments were of great value, and improved the scientific part as well as the structure of the manuscript. We hope that the revisions that have been implemented will be considered to meet the criteria needed for publication. The manuscript has been revised according to the reviewers’ suggestions. Each concern is discussed in detail and the following amendments were made:

**Reviewer: Poliana Duarte**

Major Compulsory Revisions

This study evaluated the levels of 27 biomarkers in the GCF of patients with type 2 DM and CP, only CP and only type 2 DM. Considering the high prevalence of DM and CP as well as the need of a better understanding of the molecular mechanisms involved in the pathogenesis of DM-related periodontitis, the topic of study is important. However, the paper presents major concerns, especially those related to the methods and interpretation of the results, as follows:

**Abstract**

Methods: “The material comprised...” It is better “The study population comprised...”

The sentence has been revised accordingly (Page 2, line 9)
Results: This section should be extensively revised, considering that some biomarkers were statistically similar between groups, as represented by the letters “a,b” in tables (please see the commentary in the results section). According to tables, IL-1# IL-1#, GM-CSF and IL-8 did not differ among groups. Please, revise this and other possible misinterpretation. In addition, this section should not present the tendencies and the non-significant negative correlation between the anti-inflammatory cytokines and the glycated hemoglobin levels. Please, focus on the statistically significant results after adjustments for confounders in this section.

The results section has been revised according to the reviewer suggestion by focusing on the statistically significant results (Page 2, lines 16-24).

[After adjustment for potential confounders, the DM+CP group had higher levels of IL-8 and MIP-1β, and lower levels of TNF-α, IL-4, INF-γ, RANTES and IL-7 compared to the CP group. Moreover, the DM+CP group had lower levels of IL-6, IL-7 and G-CSF compared to the DM group. The DM group had higher levels of IL-10, VEGF, and G-CSF compared to the CP group. The levels of MIP-1α and FGF were lower in diabetes patients (regardless of their periodontal status) than in chronic periodontitis subjects without diabetes. Diabetes patients (DM+CP and DM) had higher Th-2/Th-1 ratio compared to the CP group. HbA1c correlated positively with the pro-inflammatory cytokines (Pearson correlation coefficient = 0.27, P value: 0.02).]

Conclusion: This section is not coherent with the results that demonstrated that DM and CP, together and individually, may affect the GCF levels of biomarkers. Please, revise.

This section has been modified to be more specific according to the significant findings of the data (Page 3, lines 1-4).

[Type 2 diabetes and chronic periodontitis may influence the GCF levels of inflammatory molecules synergistically as well as independently. Type 2 diabetes was associated with high Th-2/Th-1 ratio, and modulated the local expression of molecules involved in the anti-inflammatory and healing processes.]

Background

First paragraph: The worldwide data of DM epidemiology should be presented instead of the Sudan data.

The worldwide data from the latest IDF (International Diabetes Federation) report in 2014 have been included in the first paragraph (Page 3, lines 10-13).

[Diabetes is a major public health concern with 380 million people suffering from the disease worldwide, and about 80% of the patients are from low- and middle-income countries (IDF report 2014). It is expected that Africa will take the lead in terms of the largest proportional increase in adults with diabetes by 2030 (Shaw JE et al., 2010).]
Last paragraph: The hypothesis should not be presented, as none inference was made regarding this hypothesis throughout the text.

We appreciate this suggestion; we followed the STROBE criteria for reporting of observational studies. “The Introduction section should describe why the study was done and what questions and hypotheses it addresses. It should allow others to understand the study’s context and judge its potential contribution to current knowledge.” (Vandenbroucke et al., 2007)

Methods

One of the major drawbacks of this paper is the criterion used to diagnosis CP (i.e. at least two sites with bleeding pockets ≥ 4mm). According to the criterion determined by the authors, CP patients may have only minor or even no periodontitis, as clinical attachment loss was not taken into consideration. Pockets without attachment loss may be attributed to excessive gingival overgrowth due to altered passive eruption or due to excessive gingival inflammation.

Thank you for highlighting this point. As the CAL was not measured, the case definition of chronic periodontitis was based on the pocket depth and BoP. As a result, there might be some sort of misclassification that might lead to underestimation of the effect of the T2DM on the study outcome (local expression of the inflammatory molecules) (Burstyn et al., 2014). This point has been elaborated as a limitation in the discussion section (Page 12, lines 21-24, and Page 13, lines 1-2).

[In addition, pocket depth and bleeding on probing were both used to define cases with periodontitis, as clinical attachment loss data were not available. Consequently, the effect of type 2 diabetes on the study outcome might be underestimated (Burstyn et al., 2014). Nevertheless, it was reported that both periodontal pocket depth and BoP reflect the current disease status and are strongly related to the local inflammatory activity compared to clinical attachment loss, which reflects past disease experience (Zhong et al., 2007, Lopez et al., 2011, Zimmermann et al., 2015).]

Calibration details were neglected.

The clinical examination was performed by a single examiner (HGM) who was trained and calibrated to perform oral examination at the Department of Clinical Dentistry-University of Bergen, Norway. Moreover, the clinical examination was repeated for 20 patients to test the intra-examiner reliability, and Kappa test was performed to calculate the Cohen's kappa coefficient.

For clarity, information about this point has been added at the end of the clinical examination section of the methodology. (Page 6, lines 20-22)
[The oral examination was repeated for 20 participants randomly selected within 2 weeks. Intra-examiner reliability was assessed by Cohen's kappa coefficient (Cohen, 1960). Kappa value ($\kappa$) was 0.88 for chronic periodontitis (yes/no).]

Another the major drawback of this paper is the absence of information regarding the characteristics of the sites selected for GCF sampling. If a patients with CP has attended to the minimum criterion (i.e. only two sites with bleeding pockets # 4mm), it means that of the four sites selected for GCF sampling in this patient, two had probing depth < 4mm. It seems that sites with totally different characteristics were pooled. Standardizing the characteristic of the selected sites among groups may exclude the possible interference of disease severity on the levels of biomarkers. Were the increased/decreased levels of biomarkers a direct consequence of the diabetic or periodontal status?

At least, two of the sites from which GCF samples were obtained in chronic periodontitis patients had PD $\geq$ 4 mm, while all the sampled sites of subjects without periodontitis ranged from 1 to 3 mm. Samples were pooled as we were not interested in site-specific results. However, the target was the individual level. In addition, it was not feasible to analyse the GCF samples per site using the multiplex assay. Therefore, pooled design of samples from the 4 quadrants was adopted (Kendziorski et al., 2005, Zhang and Gant, 2005, Kinane et al., 2011).

For a valid comparison among patients with chronic periodontitis, variations in periodontal parameters between the study groups should be minimal. Therefore, BoP was introduced as a covariant in the GLM. Moreover, there were no differences between the study groups in either the mean pocket depth, or the severity of the disease (PD 4-5 mm vs PD $\geq$ 6 mm). This information has been added to (Table 1).

The authors did not use a device to measure volume of GCF but measured the total amount of protein in each sample. Although the authors provide adjustment for the total protein in the GLM analysis, it is recommended to express each biomarker level as a proportion relative to the total protein level. The total protein should be used to normalize the data.

It was not possible to measure the GCF volume, because the device (Periotron) was not available at the field in Sudan. As a result, the levels of the molecules were expressed as (pg/30 s) (Thunell et al., 2010)

To insure a standardized method for measuring the levels of cytokines from the extracted GCF protein, the following was done:
• The time for GCF collection was standardized for all participants (30 s) (Lamster and Ahlo, 2007).
• Protein was extracted from each sample using Tween buffer (230 µl) and protein concentration of each sample was measures by BCA kit.
• Although difference between concentrations of the extracted protein was not statistically significant between the study groups, we included the protein concentration in a regression model to control for the potential effect of variability of protein concentration on the levels of the investigated biomarkers and by that; we assume that the adjusted analysis is not influenced by the variability of protein concentration if any.

Using the normal distribution for the current data was accurate based on the residuals and diagnostics. A proportion (observed amount/total protein) will not follow a normal distribution. We have re-done the analyses according to the advice from the reviewer and had odd results.

For instance, for patient 1, the IL-6 amount was 7.90 pg, and the total GCF protein for that patient was 80,000,000 pg, so the proportion is 0.0000000988.

Hence, we argue that using the present approach, and adjusting for the total protein is preferable.

Another important concern is the inclusion of smokers without statistical adjustments for this important confounder. Smoking is a well-recognized risk factor for periodontal diseases that has direct effects on the biomarker levels. The percentage of smokers in the CP group is higher when compared to the other groups.

Smoking status has been introduced in the GLM model, and the adjusted means in Table 3 have been modified accordingly.

Results

Table 1: It is pocket depth related to the full-mouth? Mean pocket depth of the DM group was not presented.

The probing depth was measured for all teeth except the 3rd molars at four sites (mesial, distal, buccal and lingual). The mean pocket depth in Table 1 represents all the diseased sites in the oral cavity with PD \( \geq 4 \) mm.

For clarity, the pocket depth was categorized to (4 – 5 mm), which represent mild to moderate periodontitis, and (\( \geq 6 \) mm) which represent those with severe form of the disease. The data have been added in Table 1.

Clinical parameters of the sampled sites were not presented.

As stated above, At least, two of the sites from which GCF samples were obtained in chronic periodontitis patients had PD \( \geq 4 \) mm, while all the sampled sites of subjects without periodontitis ranged from 1 to 3 mm. Moreover a pooled design was adopted for the GCF analysis, therefore; the site-specific data were not presented.

This section should be revised in order to describe the actual differences among groups according to the statistical analysis. For example: “The DM+CP group had the highest levels of
IL-1#, IL-8, MIP-1# and GM-CSF,...” IL-1#, GM-CSF and IL-8 did not differ among groups. MIP-1# description is not coherent with table 2. “the CP group had the highest levels of IL-4, IL-9, IL-17, TNF-#, MCP-1, MIP-1#, RANTES, FGF, PDGF, and INF-#,...” The authors should be aware that the IL-4, TNF-#, IL-17 ... levels in CP group did not differ from DM group. IL-9 did not differ among groups. “The DM group had the highest levels of IL-2, IL-6, IL-7, IL-10, IL-12, IP-10, VEGF and G-CSF...” Please, revise according to the above mentioned comments.

The results section has been extensively revised to highlight the statistically significant findings of the adjusted analysis. (Page 9, lines 13-19)

[After adjustment for potential confounders (age, gender, smoking status, BoP, dental plaque index and total protein), the DM+CP group had higher levels of IL-8 and MIP-1β, and lower levels of TNF-α, IL-4, INF-γ, RANTES and IL-7 compared to the CP group. Moreover, the DM+CP group had lower levels of IL-6, IL-7 and G-CSF than the DM group. The DM group had higher levels of IL-10, VEGF, and G-CSF than the CP group. Both diabetes groups (DM+CP and DM) had lower levels of MIP-1α and FGF compared to chronic periodontitis subjects without diabetes (CP)]

“Group analysis of the detected inflammatory...” The presentation of “tendencies” should be avoided. In addition, it is important to mention that the correlation between pro-inflammatory cytokines and the glycated hemoglobin is slight (correlation coefficient = 0.27).

This part of the results section has been revised according to the reviewer suggestions (Page 9, lines 20-24).

[The Th-2/Th-1 ratio was significantly higher in the diabetes groups (DM+CP and DM) than in the CP group (Figure 1D). A weak positive correlation was observed between HbA1c and the levels of the pro-inflammatory cytokines (Pearson correlation coefficient: 0.27, P value: 0.02) (Figure 2), while the correlation between HbA1c and the anti-inflammatory cytokines was not statistically significant (Pearson correlation coefficient: -0.11, P value: 0.33) (Figure 3).]

Discussion

The discussion section is too speculative and should be revised according to the actual statistical differences observed among groups.

This section has been revised to stress on the significant results in accordance with the changes in the results section.

First paragraph is no necessary.

The first paragraph had been deleted.
IL-6 was included as a Th-2 protein in the Th1/Th2 ratio. However, the references used in the discussion section for the statement that this cytokine is multifunctional presenting pro- and anti-inflammatory activities are not appropriated. Please, revise.

The citation for IL-6 as a multifunctional cytokines has been updated (Kishimoto T: Interleukin-6: discovery of a pleiotropic cytokine. Arthritis research & therapy 2006, 8 Suppl 2:S2.) (Page 10, lines 20-22).

“In the present study, the level of IL-1# was highest in the DM+CP group.” This statement is not coherent with the tables.

The mean level of IL-1β was (350.50 pg/30s) in the DM+CP, compared to (260.31) in the CP group and (261.74) in the DM group, and the results were not statistically significant.

For clarity, the sentence has been modified (Page 10, lines 4-5).

[In the present study, the level of IL-1β was highest in the DM+CP group, albeit not statistically significant.]

“This is in accordance with a study which reported a significant positive correlation between IL-1# levels in GCF and HbA1c [34]. The mentioned study (34) only studied the IL-1#.” Therefore, it is not in accordance with the present study.

It appears that the way the statement was written is a bit confusing. For clarity, the sentence in Page 10, lines 18-19 has been modified:

[Another study reported a significant positive correlation between IL-1β levels in GCF and HbA1c (Engebretson et al., 2004).]

“This process might be disrupted in patients having both T2DM and periodontitis, as indicated by the lower levels of IL-6 in the DM+CP group than in the DM and CP groups.” IL-6 levels were similar between CP and DM groups.

The difference in IL-6 levels between the DM+CP group and the CP group was not statistically significant. Therefore, this statement was omitted form the discussion part.

Conclusion is too speculative and not coherent with the findings of the study. This section is not coherent with the results that demonstrated that DM and CP, together and individually, may affect the GCF levels of biomarkers. Please, revise.

The conclusion has been rewritten to be more specific and in accordance with the changes in the results section (Page 13, lines 4-7).
[Type 2 diabetes and chronic periodontitis may adversely influence the GCF levels of inflammatory molecules synergistically as well as independently. Moreover, type 2 diabetes was associated with high Th-2/Th-1 ratio, and adversely influenced the local expression of molecules involved in the anti-inflammatory and healing processes.]

Reviewer: Fawad Javed

This manuscript addresses an interesting issue but needs major revisions before it can be considered further. I regret to say that the manuscript is carelessly written and many important references have been skipped.

The references list has been updated to include more references in the field.

First of all, the title does not match with the objectives. Let me remind you that the aim, conclusions and title should match.

Thank you for the information. The conclusion has been revised to be more specific (Page 13, lines 4-7), and to match the title (Influence of type 2 diabetes on local production of inflammatory molecules in adults with and without chronic periodontitis), and the aim (to investigate the effect of T2DM on the local expression of inflammatory molecules involved in periodontal inflammation and healing).

Moreover, your title is incomplete. From a well-written title, the reader should be able to understand the objectives of a study.

Thank you for this insightful comment. The title was constructed according to the STROBE criteria and to meet the *BMC-Oral Health* criteria (Vandenbroucke et al., 2007).

There are undefined abbreviations throughout the manuscript. What is the difference between DM and T2DM?

The abbreviation (T2DM) has been omitted throughout the text and replaced by “type 2 diabetes”. All the other abbreviations were clarified in the abbreviations section (Page 13, lines 11-18). Regarding the abbreviation (DM) it was defined the first time it appeared in the text (Page 2, line 11) as study participants with T2DM without periodontitis. The same abbreviation (DM) for this specific group was used by others (Sun WL et al., 2011) (Longo et al., 2014) (Kardesler et al., 2008) (Trivedi et al., 2014).

The results in the abstract reflect that HbA11c levels were assessed but your methodology does not mention that this parameter was assessed. Please read and revise your abstract. Methodology is also poorly expressed in the abstract.
The abstract has been revised and the following sentence has been added to the methods part of the abstract according to the reviewer suggestion (Page 2, lines 14-15):

[A glycated haemoglobin (HbA1c) test was performed for patients with diabetes by boronate affinity chromatography.]

In addition, this information has been updated in the methods section of the manuscript (Page 5, lines 16-18):

[Whole blood samples obtained from patients with diabetes were analysed for HbA1c by boronate affinity chromatography using a commercially available kit (LabonaCheckTM A1c analyzer) (Little and Roberts, 2009).]

The introduction merely repeats well known facts and fails to explain what is lacking or needs to be known/clarified in this area of research.

Thank you for pointing out this part. The introduction started with general information about diabetes, chronic periodontitis and epidemiological data about the diseases. Then it touched upon some of the mechanism by which diabetes affects periodontal tissues.

The gap in the current knowledge was stated in (Page 4, lines 19-24). There is a need for comprehensive analysis of the molecules involved in the inflammatory milieu as most of the studies in the field investigated a limited number of inflammatory molecules. Using multiplex technique in the present study allowed us to develop a global picture of the inflammatory process involved in both diabetes and periodontal disease by investigating a relatively wide range of inflammatory molecules.

There is no hypothesis either.

The hypothesis was stated in at the end of the introduction section (Page 5, lines 5-7)

[We tested the hypothesis that type 2 diabetes adversely influences the local expression of the inflammatory molecules under investigation.]

The methodology is poorly expressed. How was chronic periodontitis defined?

The case definition was stated in the methods section under the sub-heading (clinical periodontal examination) (Page 6, lines 18-20).

[Participants were diagnosed as having chronic periodontitis if they had at least two sites with bleeding pockets of ≥ 4 mm (not on the same tooth) (Katagiri et al., 2012, Eke et al., 2012).]

In addition the case definition of periodontitis was discussed in the discussion section (Page 12, lines 21-24, and Page 13, lines 1-2)
[In addition, probing depth and bleeding on probing were both used to define cases with periodontitis, as clinical attachment loss data were not available. Consequently, the effect of type 2 diabetes on the study outcome might be underestimated (Burstyn et al., 2014). Nevertheless, it was reported that both periodontal pocket depth and BoP reflect the current disease status and are strongly related to the local inflammatory activity compared to clinical attachment loss, which reflects past disease experience (Zhong et al., 2007, Lopez et al., 2011, Zimmermann et al., 2015).]

Various parameters (such as chronic periodontitis, measurement of glycemic levels and demographic characteristics of the study population are mixed up. Use of separate subheadings could have eased comprehension and could have given a flow to this manuscript.

The methods part has 6 sub-headings:

- Study design and participants (Page 5, line 9),
- Clinical periodontal examination (Page 6, line 10),
- GCF sampling (Page 7, line 1),
- Protein extraction and quantification (Page 7. Line 12),
- Analysis and grouping of inflammatory molecules (Page 7, line 19) and
- Statistical analysis (Page 8, line 18).

How was the sample size estimated? Why didn’t the authors perform a power analysis?

We appreciate this observation. This study was based on sub-sample of a previous study (Mohamed et al., 2013). For that study, a sample size calculation was performed.

Furthermore, our sample size for this study is comparable with other studies addressing similar issues (Duarte et al., 2014), (Javed et al., 2015), (Pradeep et al., 2011).

In addition, calculation of post.hoc power is in general statistically not recommended.

http://amstat.tandfonline.com/doi/pdf/10.1198/000313001300339897


For clarity, the following sentence has been added to the methods section (Page 5, lines 10-11):

[In all, 108 individuals were enrolled in the study, representing a randomly selected subset from 461 participants recruited for a previous study by Mohamed et al. (Mohamed et al., 2013).]

The results are presented in a very complicated manner......very difficult to interpret.

This section has been extensively revised to be more focused on the statistically significant results (Page 9, lines 12-19).
[After adjustment for potential confounders (age, gender, smoking status, BoP, dental plaque index and total protein), the DM+CP group had higher levels of IL-8 and MIP-1β, and lower levels of TNF-α, IL-4, INF-γ, RANTES and IL-7 compared to the CP group. Moreover, the DM+CP group had lower levels of IL-6, IL-7 and G-CSF than the DM group. The DM group had higher levels of IL-10, VEGF, and G-CSF than the CP group. Both diabetes groups (DM+CP and DM) had lower levels of MIP-1α and FGF compared to chronic periodontitis subjects without diabetes (CP)].

In the discussion, the authors have strived hard to support their results by comparing them with other studies.

The discussion section has been revised according to the changes in the results section. We tried to follow the STROBE criteria while constructing this section. (Vandenbroucke et al., 2007)

It remains unclear what this study adds. All facts reported here are already known. What is new here?

This study is the first to report information about T2DM patients in Sudan and the nearby geographical area, and some of the trends that we have observed need to be investigated further. Moreover, there is an urgent need for interventions to tackle the problem in this region (Bos and Agyemang, 2013).

In addition, to our knowledge, this study is the first to investigate GCF levels of IL-1ra, IL-5, IL-9, IL-13, IL-15, FGF, IP-10, MIP-1β, PDFG and RANTES in patients with type 2 diabetes.

What are the limitations of this work?

For clarity, the limitations have been revised and stated at the end of the discussion section (Page 12, lines 18-24, and Page 13, lines 1-2).

[In the present study, the GCF volume was not measured. Therefore, total GCF protein was used as a surrogate measure of the GCF volume in the multivariate analysis as an attempt to control for the potential effect of variability of GCF volume on our results (Petropoulos et al., 2004). In addition, pocket depth and bleeding on probing were both used to define cases with periodontitis, as clinical attachment loss data were not available. Consequently, the effect of type 2 diabetes on the study outcome might be underestimated (Burstyn et al., 2014). Nevertheless, it was reported that both periodontal pocket depth and BoP reflect the current disease status and are strongly related to the local inflammatory activity compared to clinical attachment loss, which reflects past disease experience (Zhong et al., 2007, Lopez et al., 2011, Zimmermann et al., 2015).]
What are the future perspectives?

The future perspectives were stated in the conclusions section (Page 13, lines 7-9)

[Further prospective studies are warranted to produce sufficient evidence to support the application of specific GCF biomarkers for prediction and prognosis of periodontal disease among patients with diabetes.]

Would there be a difference in nonsurgical periodontal therapy is performed in chronic periodontitis patients with and without diabetes?

Thank you for raising this important point. Most of the follow-up studies that performed nonsurgical periodontal therapy as an intervention focused on metabolic control as an outcome (Simpson et al., 2010, Teeuw WJ, 2010 Feb, Nesse et al., 2009, Engebretson and Kocher, 2013, Wang et al., 2014). However, most of the studies that investigated the effect of periodontal therapy on inflammatory cytokines reported similar response to treatment in subjects with and without diabetes (Correa et al., 2010, Sun et al., 2010, Kardesler et al., 2010). We did not include information about the effect of periodontal therapy on the local expression of inflammatory molecules because it is out of the scope of the present study, but it would be of great value to investigate that in the future.

Can these immunological markers be used as diagnostic tools for detection of diabetes?

A single prognostic biomarker must have the highest levels of sensitivity and specificity for disease progression. Therefore, the interest has been shifted towards considering combinations of different host responses to develop a global view of the inflammatory process, which is one of the strengths of this report (Kinney et al., 2014, Hanes and Krishna, 2010, Barnes et al., 2014).

The following sentences have been added to the discussion section according to the reviewer suggestion (Page 12, lines 16-18):

[Up to date, there is no ideal biomarker that can be nominated for disease detection or progression. Therefore, the interest has been shifted towards considering combinations of various host responses (Kinney et al., 2014, Hanes and Krishna, 2010)].

In addition, the molecules that have the potentials to be used as biomarkers for T2DM diagnosis such as ghrelin, leptin and visfatin have been investigated in a another study (Mohamed et al., 2015, in press).
References:


